



Attorney Docket No. A8709

**METHODS FOR DIAGNOSING A BIPOLAR DISORDER AND  
UNIPOLAR DISORDER**

This application claims the benefit of U.S. Provisional Application No. 60/515,846, filed October 30, 2003, incorporated herein by reference.

5

**Field of the Invention**

The present invention relates to a method for diagnosing a bipolar disorder. In particular, this method utilizes changes that occur in  $\text{Na}^+ \text{K}^+$  ATPase regulation in cells from bipolar individuals, as compared to cells from unaffected control individuals, to provide a diagnostic assay for a bipolar disorder. The present invention also relates to a method for diagnosing unipolar disorder.

15

**Background of the Invention**

Mental illness afflicts nearly ten percent of the general population both in the United States and in the rest of the world. Bipolar (manic depressive) disorders occur ~~disorder occurs~~ in one to two percent of the population and are ~~is~~ the sixth leading cause of disability (Coryell et al., Am. J. Psychiatry 150:720-727 (1993); Lopez, A.D., and Murray, C.C., Nat. Med. 4:1241-1243 (1998); Hyman, S.E., Am. J. Geriatr. Psychiatry 9:330-339 (2001)). A problem facing the medical community is misdiagnosis of a bipolar disorder. Misdiagnosed patients receive an average of 3.5 misdiagnoses and consult four physicians before receiving an accurate diagnosis ("Living with bipolar disorder: How far

have we really come?" National Depressive and Manic-Depressive Association, Chicago, IL (2001)).

The current diagnostic method for a bipolar disorder (bipolar disorder I and II) involves a series of clinical interviews and examination using the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV), the main diagnostic reference of Mental Health professionals in the United States, which is now in its fourth edition. Significant controversy exists about the validity of this manual, which limits the accuracy of clinical diagnosis (Torrey et al, "Surviving Manic Depression", *Basic Books*, New York (2002)). In addition, attempts are underway to identify the genes underlying these illnesses ~~this illness~~ and thereby develop diagnostic markers. However, their identification and possible use as diagnostic markers are years away (Bradbury, J., *Lancet* 357:1596 (2001)).

The lifetime risk for unipolar disorder (major depressive disorder) is 10% to 25% for women and from 5% to 12% for men. At any point in time, 5% to 9% of women and 2% to 3% of men suffer from this disorder. Prevalence is unrelated to ethnicity, education, income, or marital status.

Like bipolar disorders ~~disorder~~, unipolar disorder is also currently diagnosed using the DSM-IV. By definition, unipolar disorder and bipolar disorders ~~disorder~~ are distinct conditions. Unipolar disorder is diagnosed when there has never been a manic episode and at least five of the following symptoms have been present during the same 2 week depressed period:

- Abnormal depressed mood.
- Abnormal loss of all interest and pleasure.
- Appetite or weight disturbance, either:
  - Abnormal weight loss (when not dieting) or decrease in appetite.
  - Abnormal weight gain or increase in appetite.
- Sleep disturbance, either abnormal insomnia or abnormal hypersomnia.
- Activity disturbance, either abnormal agitation or abnormal slowing (observable by others).
- Abnormal fatigue or loss of energy.
- Abnormal self-reproach or inappropriate guilt.
- Abnormal poor concentration or indecisiveness.
- Abnormal morbid thoughts of death or suicide.

There is evidence that unipolar disorder is, in part, a genetic disorder. Therefore, as with bipolar disorders, attempts are underway to identify the genes underlying unipolar disorder and thereby develop diagnostic markers. However, this has yet to be achieved.

In virtually every animal cell, the concentration of  $\text{Na}^+$  in the cell (~12 mM) is lower than the concentration of  $\text{Na}^+$  in the surrounding medium (~145 mM), and the concentration of  $\text{K}^+$  in the cell (~140 mM) is higher than the concentration of  $\text{K}^+$  in the surrounding medium (~4 mM). This imbalance is established and maintained by an active transport system in the plasma membrane. The transporter enzyme  $\text{Na}^+ \text{K}^+$  ATPase, also known as the sodium pump, couples breakdown of ATP to the simultaneous movement of both  $\text{Na}^+$  and  $\text{K}^+$  against their electrochemical gradients. For each molecule of ATP

hydrolyzed to ADP and  $P_i$ , the  $Na^+K^+$  ATPase transports two  $K^+$  ions inward and three  $Na^+$  ions outward across the plasma membrane.

The  $Na^+K^+$  ATPase is an integral protein with two subunits ( $M_r \sim 50,000$  and  $\sim 110,000$ ), both of which span the membrane. A proposed mechanism by which ATP hydrolysis is coupled to ion transport involves the  $Na^+K^+$  ATPase cycling between two forms, a phosphorylated form with high affinity for  $K^+$  and low affinity for  $Na^+$ , and a dephosphorylated form with high affinity for  $Na^+$  and low affinity for  $K^+$ . The conversion of ATP to ADP and  $P_i$  occurs in two steps catalyzed by the enzyme.

In addition to the  $Na^+K^+$  ATPase, the plasma membrane also contains channel proteins that allow the principal cellular ions ( $Na^+$ ,  $K^+$ ,  $Ca^{2+}$ , and  $Cl^-$ ) to move through them at different rates down their concentration gradients. Ion concentration gradients generated by pumps and selective movement of ions through channels constitutes the principal mechanism by which a difference in voltage, or electric potential, is generated across the plasma membrane. However, because the plasma membranes of animal cells contain many open  $K^+$  channels, and relatively few open  $Na^+$ ,  $Ca^{2+}$ , and  $Cl^-$  channels, the membrane potential in animal cells depends largely on open  $K^+$  channels. As a result, the major ionic movement across the plasma membrane is that of  $K^+$  from the inside outward, powered by the  $K^+$  concentration gradient, leaving an excess of negative charge on the inside and creating an excess of positive charge on the outside.

The magnitude of this membrane potential generally is -50 mV to -70 mV (with the inside of the cell negative relative to the outside), which is characteristic of most animal cells and essential to 5 the conduction of action potentials in neurons. As noted earlier, the K<sup>+</sup> concentration gradient that drives the flow of K<sup>+</sup> ions through open K<sup>+</sup> channels is generated by the Na<sup>+</sup>K<sup>+</sup> ATPase. The central role of the Na<sup>+</sup>K<sup>+</sup> ATPase is reflected in the energy invested 10 in this reaction: about 25% of the total energy consumption of a human at rest.

The steroid derivative ouabain is a potent and specific inhibitor of the Na<sup>+</sup>K<sup>+</sup> ATPase. Ouabain and another steroid derivative, digitoxigenin, are the active ingredients of digitalis, which has long been 15 used to treat congestive heart failure. Inhibition of the Na<sup>+</sup>K<sup>+</sup> ATPase by digitalis leads to an increased Na<sup>+</sup> concentration in cells, activating a Na<sup>+</sup>Ca<sup>2+</sup> antiporter in cardiac muscle. The increased 20 influx of Ca<sup>2+</sup> through this antiporter produces elevated cytosolic Ca<sup>2+</sup>, which strengthens the contractions of heart muscle.

The Na<sup>+</sup>K<sup>+</sup> ATPase has also been investigated for its possible involvement in bipolar disorder 25 pathophysiology (El-Mallakh et al, *Biol. Psychiatry*, 537:235-244 (1995)). However, this has been an unsettled and controversial subject in the field for many years. Na<sup>+</sup>K<sup>+</sup> ATPase activity has been variously reported to be increased, decreased, or unchanged in 30 bipolar patients. In 1997, Looney et al conducted a meta-analysis of the available literature on erythrocyte Na<sup>+</sup>K<sup>+</sup> ATPase activity in bipolar disorders disorder and concluded that it is lower in | disorders disorder

bipolar patients (Looney et al, *Depress. Anxiety*, 5:53-65 (1997)). However, the question of exactly how the  $\text{Na}^+\text{K}^+$  ATPase plays a role in bipolar disorders disorder remains unanswered.

Lithium, an alkaline metal that has been used successfully for over fifty years to stabilize mood in bipolar disorders disorder, has been shown to augment  $\text{Na}^+\text{K}^+$  ATPase activity. Recently, the role of lithium in depolarizing the resting membrane potential of neurons has been analyzed (Thiruvengadam, *J. Affect. Disord.*, 65:95-99 (2001); and Thiruvengadam, "Electro-biochemical coupling, excitability of neurons and bipolar disorder, *Bipolar Disorder* 3 (2001)). Hyperpolarization of membrane potential in leukocytes of bipolar patients and depolarization following the addition of lithium has been observed (El Mallakh et al, *J. Affect. Disord.*, 41:33-37 (1996)). In addition, a significantly smaller increase in  $\text{Na}^+\text{K}^+$  ATPase density after incubation for 72 hours in ethacrynat or lithium has been observed in cells of bipolar patients compared to cells of unaffected individuals (Wood et al, *J. Affect. Disord.*, 21:199-206 (1991)).

El-Mallakh et al measured the transmembrane potential in leukocytes from hospitalized bipolar patients and observed that the transmembrane potential of the bipolar patients was hyperpolarized compared with normal controls and euthymic patients on lithium (El-Mallakh et al, *J. Affect. Disord.*, 41:33-37 (1996)). However, Tamella-Buss et al measured the membrane potentials of cultured lymphoblasts and concluded that there was no significant difference in membrane potentials among

bipolar patients, their siblings and normal controls (Buss Tamella et al, *Psychiatry Res.* 59:197-201 (1996)).

In view of the previously studies on the 5 possible involvement of the  $\text{Na}^+ \text{K}^+$  ATPase in bipolar disorders disorder, one would not expect  $\text{Na}^+ \text{K}^+$  ATPase activity to serve as a reliable basis for diagnosing a bipolar disorder in an individual patient, because measurements of  $\text{Na}^+ \text{K}^+$  ATPase activity are highly 10 variable. Similarly, one would not expect transmembrane potential to serve as a reliable basis for diagnosing a bipolar disorder in an individual patient, because measurements of transmembrane potential are highly variable.

Accordingly, despite the existence of 15 treatments for bipolar disorders disorder and unipolar disorder and recent advances in the psychiatric field, there remains a heretofore unmet need for clinical tests to augment the DSM-IV in 20 diagnosing bipolar disorders disorder and unipolar disorder.

#### Summary of the Invention

The present invention exploits changes that 25 take place in  $\text{Na}^+ \text{K}^+$  ATPase regulation in cells of bipolar patients, as compared to cells of normal, unaffected control subjects, to provide a diagnostic assay for a bipolar disorder. The present invention provides a reliable diagnostic assay to differentiate 30 cells from bipolar patients from those of normal, unaffected individuals, schizophrenic individuals, and unipolar (depressive) individuals. The diagnostic assay of the invention may also or instead exploit

the similarity of cells from bipolar patients to those of people already known to have a bipolar disorder. Thus, the present invention addresses the heretofore unmet need for a clinical test useful in 5 | the diagnosis of a bipolar disorder.

The present invention further provides a diagnostic assay useful in differentiating cells from unipolar (depressive) patients from those of normal, unaffected individuals, schizophrenic individuals, 10 and bipolar individuals. This diagnostic assay may also or instead exploit the similarity of cells from unipolar patients to those of people already known to have unipolar disorder.

As shown herein, the membrane potential in 15 cultured cells from bipolar patients is significantly different than the membrane potential in cultured cells from unaffected controls and siblings. For example, the membrane potentials of bipolar lymphoblasts are significantly hyperpolarized 20 when compared with those of siblings and negative controls. The changes in membrane potential reflect changes in  $\text{Na}^+ \text{K}^+$  ATPase regulation that occur in cells of patients affected with a bipolar disorder.

In preferred embodiments, a diagnostic assay 25 | for a bipolar disorder according to the invention utilizes changes in membrane potential in lymphoblasts or whole blood cells; however, a diagnostic assay for a bipolar disorder according 30 | to the invention can also employ changes in membrane potential in lymphocytes, erythrocytes, as well as in other cells.

In additional embodiments,  $\text{Na}^+ \text{K}^+$  ATPase activity and therefore membrane potential is manipulated by

incubating cells in K<sup>+</sup>-free buffer and/or with one or more compounds that alter the Na<sup>+</sup> and K<sup>+</sup> ionic gradients in the cells. Comparisons between cells from bipolar patients and control cells from 5 unaffected individuals (negative controls) incubated under corresponding conditions reveal significant differences that can be quantified and used to diagnose a bipolar disorder. Likewise, comparisons between cells from bipolar patients and cells from 10 other individuals known to have a bipolar disorder (positive controls) incubated under corresponding conditions reveal a lack of a significant difference that can be used to diagnose a bipolar disorder.

Unipolar disorder may be diagnosed in a similar 15 manner. For example, comparisons between cells from unipolar patients and control cells from unaffected individuals (negative controls) incubated under corresponding conditions reveal significant differences that can be quantified and used to 20 diagnose unipolar disorder. Likewise, comparisons between cells from unipolar patients and cells from other individuals known to have unipolar disorder incubated under corresponding conditions reveal a lack of a significant difference that can be used to 25 diagnose unipolar disorder.

In a preferred embodiment, a diagnostic assay 30 for unipolar disorder according to the invention utilizes changes in membrane potential in whole blood cells; however, a diagnostic assay for unipolar disorder can also employ changes in membrane potential in lymphoblasts, lymphocytes, erythrocytes, as well as in other cells.

The membrane potential of a patient's cells can be ascertained by any conventional method, such as by measuring the fluorescence intensity of a lipophilic fluorescent dye. For example, membrane potentials may be measured using 3,3'-dihexyloxacarbocyanine iodide DiOC<sub>6</sub>(3), a cell-permeant, voltage sensitive, green-fluorescent dye, in conjunction with a fluorescence spectrometer.

10

#### Brief Description of the Drawings

Figure 1 shows the membrane potentials of affected (bipolar\_I) lymphoblasts, non-affected sibling lymphoblasts, and normal lymphoblasts, as indicated by fluorescence intensity of DiOC<sub>6</sub>(3) (7 nM), in regular buffer containing K<sup>+</sup>.

15

Figure 2 shows the membrane potentials of affected (bipolar\_I) cells and unaffected cells, as indicated by the fluorescence intensity of DiOC<sub>6</sub>(3) in affected cells and the combination of non-affected sibling cells and normal cells.

20

Figure 3 shows the fluorescence intensity of DiOC<sub>6</sub>(3) in affected (bipolar\_I) cells and unaffected (sibling and normal) cells relative to the fluorescence intensity of DiOC<sub>6</sub>(3) in normal control cell N1.

25

Figure 4 shows a comparison of membrane potentials in K<sup>+</sup>-free buffer and in buffer containing K<sup>+</sup>, as indicated by the ratio of fluorescence intensity of DiOC<sub>6</sub>(3) in affected (bipolar\_I) cells and unaffected (sibling and normal) cells in K<sup>+</sup>-free buffer and in K<sup>+</sup>-containing (regular) buffer.

30

Figure 5 shows ethacrylate-induced changes in membrane potential, as indicated by the relative

ratio of the fluorescence intensity and therefore of  
the membrane potential of affected (bipolar\_I) cells  
and unaffected (sibling and normal) cells in K<sup>+</sup>-free  
buffer and in K<sup>+</sup>-containing (regular) buffer in the  
5 presence or absence of 30  $\mu$ M ethacrynat.

Figure 6 shows the effect of ethacrynat on  
membrane potential, as indicated by the ratio of  
fluorescence intensity of affected (bipolar\_I) cells  
and unaffected (sibling and normal) cells in K<sup>+</sup>-free  
10 buffer with or without the addition of 30  $\mu$ M  
ethacrynat.

Figure 7 shows the relative rate of  
polarization of affected (bipolar\_I) cells and  
unaffected (sibling and normal) cells incubated for  
15 30 minutes in K<sup>+</sup>-free buffer and in K<sup>+</sup>-containing  
(regular) buffer in the presence or absence of 30  $\mu$ M  
ethacrynat.

Figure 8 shows the effect of monensin on  
membrane potential, as indicated by the ratio of  
20 fluorescence intensity of affected (bipolar\_I) cells  
and unaffected (sibling and normal) cells in K<sup>+</sup>-free  
buffer with or without the addition of 10  $\mu$ M  
monensin.

Figure 9 shows the effect of phorbol 12-  
25 myristate 13-acetate (PMA) on the rate of  
repolarization, as indicated by the ratio of  
repolarization rate of affected (bipolar\_I) cells  
and unaffected (sibling and normal) cells in K<sup>+</sup>-free  
buffer with or without 2  $\mu$ M PMA.

30 Figure 10 shows the effect of PMA on the  
relative ratio of repolarization rates, as indicated  
by the ratio of repolarization rate of affected  
(bipolar\_I) cells and unaffected (sibling and

normal) cells in both K<sup>+</sup>-free buffer and in K<sup>+</sup>-containing (regular) buffer with or without 2 μM PMA.

Figure 11 shows the effect of lithium on membrane potential, as indicated by the relative ratio of the fluorescence intensity of affected (bipolar I) cells and unaffected (sibling and normal) cells in K<sup>+</sup>-containing (regular) buffer and in K<sup>+</sup>-free buffer in the presence or absence of 20 mM lithium chloride.

Figure 12 shows the ratio of fluorescence intensities and therefore membrane potentials in K<sup>+</sup>-free buffer containing 30 μM ethacrynatet to the fluorescence intensity in K<sup>+</sup>-containing buffer without ethacrynatet in an open clinical trial using whole blood samples.

Figure 13 shows a comparison of ethacrynatet and sorbitol in K<sup>+</sup>-free buffer, as used in the open clinical trial using whole blood samples.

Figure 14 shows the ratio of fluorescence intensities and therefore membrane potentials in K<sup>+</sup>-free buffer containing 30 μM ethacrynatet to the fluorescence intensity in K<sup>+</sup>-containing buffer without ethacrynatet in a blind clinical trial using whole blood samples from 18 normal controls, 10 schizophrenics, 11 unipolars, and 20 bipolars.

Figure 15 provides an example of the use of ANOVA in the diagnosis of a representative patient ("Patient A") as bipolar. The ratio of fluorescence intensities and therefore membrane potentials in K<sup>+</sup>-free buffer containing 30 μM ethacrynatet was compared to the fluorescence intensity in K<sup>+</sup>-containing buffer without ethacrynatet.

Detailed Description of the Invention

The present invention provides a method for diagnosing a patient with a bipolar disorder by comparing the membrane potential of cells of the patient with corresponding control cells of one or more people known not to have a bipolar disorder (negative controls) and/or corresponding bipolar control cells of one or more people known to have a bipolar disorder (positive controls). In experiments described herein, the membrane potentials of lymphoblasts and whole blood cells are ascertained and compared. However, a diagnostic assay according to the present invention can utilize any cell type, such as, but not limited to, erythrocytes, platelets, leukocytes, macrophages, monocytes, dendritic cells, fibroblasts, epidermal cells, mucosal tissue cells, cells in the cerebrospinal fluid, hair cells, etc. Cells present in blood, skin cells, hair cells, or mucosal tissue cells may be more convenient to use because of the ease of harvesting these cell types.

For purposes of a diagnostic assay for a bipolar disorder disclosed herein, "corresponding control cells" means that the cells of the patient are the same type of cells as the control cells of the people known to not have a bipolar disorder. Similarly, for purposes of a diagnostic assay for unipolar disorder disclosed herein, "corresponding control cells" means that the cells of the patient are the same type of cells as the control cells of the people known to not have unipolar disorder. For example, if the cells of the patient are lymphoblasts, the "corresponding control cells" are

lymphoblasts. Likewise, if the cells of the patient are whole blood cells, the "corresponding control cells" are whole blood cells.

For purposes of a diagnostic assay for a bipolar disorder disclosed herein, "corresponding bipolar control cells" means that the cells of the patient are the same type of cells as the bipolar control cells of the people known to have a bipolar disorder. For example, if the cells of the patient are lymphoblasts, the "corresponding bipolar control cells" are lymphoblasts. Likewise, if the cells of the patient are whole blood cells, the "corresponding bipolar control cells" are whole blood cells.

For purposes of a diagnostic assay for unipolar disorder disclosed herein, "corresponding unipolar control cells" means that the cells of the patient are the same type of cells as the unipolar control cells of the people known to have unipolar disorder. For example, if the cells of the patient are whole blood cells, the "corresponding unipolar control cells" are whole blood cells. Likewise, if the cells of the patient are lymphoblasts, the "corresponding unipolar control cells" are lymphoblasts.

In a preferred embodiment, a diagnostic assay according to the present invention involves manipulating the membrane potential in cells by either incubating cells with a compound that alters ATPase activity and/or by incubating cells in potassium-free media. The membrane potential of the cells is then ascertained following such treatment.

As indicated above, a cell's membrane potential is the result of the different concentration of ions on either side of the membrane. The activity of the  $\text{Na}^+\text{K}^+$  ATPase pump, which regulates the concentration of  $\text{Na}^+$  and  $\text{K}^+$  to maintain homeostasis, can be altered by a variety of external stimuli, including various chemicals. When the  $\text{Na}^+$  and  $\text{K}^+$  ionic gradients are modulated by some means, the cell regulates the activity of the  $\text{Na}^+\text{K}^+$  ATPase in an effort to return the ionic gradients to normal levels. Some compounds, such as ethacrynone, monensin, and monensin decyl ester, alter the activity of the  $\text{Na}^+\text{K}^+$  ATPase by increasing the intracellular levels of sodium. Other compounds, such as phorbol 12-myristate 13-acetate (PMA), 12-O-tetradecanoylphorbol 13-acetate, phorbol 12-myristate 13-acetate 4-O-methyl ether, phorbol 12,13-dibutyrate (PDBu), phorbol 12,13-didecanoate (PDD), phorbol 12,13-dinonanoate 20-homovanillate, and other phorbol esters alter the activity of the  $\text{Na}^+\text{K}^+$  ATPase by increasing the density of the  $\text{Na}^+\text{K}^+$  ATPase on the cell surface. Thus, the activity of the  $\text{Na}^+\text{K}^+$  ATPase is affected by its structure, its density, and compounds (both endogenous and exogenous) that affect the structure and density. Genes may play a role also.

Table 1 below shows other examples of compounds that alter the activity of the  $\text{Na}^+\text{K}^+$  ATPase, either indirectly by altering the  $\text{K}^+$  and/or  $\text{Na}^+$  ionic gradients or by acting on the  $\text{Na}^+\text{K}^+$  ATPase itself.

TABLE 1

| <u>Chemical</u>  | <u>K<sup>+</sup></u> | <u>Na<sup>+</sup></u> | <u>K<sup>+</sup> &amp; Na<sup>+</sup></u> | <u>Na<sup>+</sup>K<sup>+</sup> ATPase</u> |
|------------------|----------------------|-----------------------|---|---|
| Valinomycin      | X                    |                       |   |   |
| Monensin         |                      | X                     |   |   |
| Gramicidin       |                      | X                     |   |   |
| PCMBS            |                      | X                     |   |   |
| Veratridine      |                      | X                     |   |   |
| Ethacrynatate    |                      |                       | X   |   |
| PMA              |                      |                       |   | X   |
| Dopamine         |                      |                       |   | X   |
| Catacholamines   |                      |                       |   | X   |
| Phorbol Esters   |                      |                       |   | X   |
| Ouabain          |                      |                       |   | X   |
| Lithium          | X                    | X                     | X   | X   |
| Valproate        |                      |                       |   | X   |
| Lamotrigine      |                      |                       |   | X   |
| Cocaine          |                      |                       | X   |   |
| Nicotine         |                      | X                     |   |   |
| R0-31-8220       |                      |                       |   | X   |
| Oxymetazoline    |                      |                       |   | X   |
| Calcineurin      |                      |                       |   | X   |
| Topiramate       |                      |                       |   | X   |
| Peptide Hormones |                      |                       |   | X   |
| Sorbitol         |                      |                       |   |   |
| Diuretics        |                      |                       | X   |   |

5        In several embodiments, an assay according to  
           the present invention employs such Na<sup>+</sup>K<sup>+</sup> ATPase-altering compounds to help diagnose patients with a  
           bipolar disorder. In other embodiments, an assay according to the present invention employs such Na<sup>+</sup>K<sup>+</sup>  
 10      ATPase-altering compounds to help diagnose patients with unipolar disorder.

15      The compounds that are described herein are merely examples of the compounds that could be used to alter Na<sup>+</sup>K<sup>+</sup> ATPase activity. For example, any compound that increases the density and/or activity of the Na<sup>+</sup>K<sup>+</sup> ATPase can be used in a diagnostic assay according to the present invention.

In another embodiment, a compound that decreases the density and/or activity of the  $\text{Na}^+\text{K}^+$  ATPase may be used in a diagnostic assay according to the present invention. For example, low concentrations of ouabain may be useful in differentiating bipolar cells from normal cells.

Thus, for purposes of this disclosure, "alters  $\text{Na}^+\text{K}^+$  ATPase activity" includes directly altering  $\text{Na}^+\text{K}^+$  ATPase activity by acting directly upon the  $\text{Na}^+\text{K}^+$  ATPase as well as indirectly altering  $\text{Na}^+\text{K}^+$  ATPase activity by, for example, increasing the intracellular sodium concentration. Furthermore, "alters  $\text{Na}^+\text{K}^+$  ATPase activity" includes increasing or decreasing  $\text{Na}^+\text{K}^+$  ATPase activity, although increasing  $\text{Na}^+\text{K}^+$  ATPase activity is preferred.

Potassium uptake in cells of bipolar patients is significantly reduced compared to potassium uptake in cells of normal, unaffected patients. In several embodiments of the present invention, the membrane potential of cells incubated in potassium-free buffer is ascertained with or without incubation with compounds that alter the activity of the  $\text{Na}^+\text{K}^+$  ATPase.

Examples of buffers that may be used in a diagnostic assay according to the present invention, along with their useful pH ranges, are shown in Table 2 below.

TABLE 2

| <u>Composition</u>                  | <u>Lower pH</u> | <u>Upper pH</u> |
|-------------------------------------|-----------------|-----------------|
| Glycyl-glycine-piperazine-2HCl-NaOH | 4.4             | 10.8            |
| MES-NaOH-NaCl                       | 5.2             | 7.1             |
| TRIS-malic acid-NaOH                | 5.2             | 8.6             |
| MES-NaOH                            | 5.6             | 6.8             |
| ADA-NaOH-NaCl                       | 5.6             | 7.5             |
| ACES-NaOH-NaCl                      | 5.9             | 7.8             |
| ACES-NaOH-NaCl                      | 5.9             | 7.8             |
| BES-NaOH-NaCl                       | 6.2             | 8.1             |
| MOPS-NaOH-NaCl                      | 6.25            | 8.15            |
| TES-NaOH-NaCl                       | 6.55            | 8.45            |
| MOPS-KOH                            | 6.6             | 7.8             |
| HEPES-NaOH-NaCl                     | 6.6             | 8.5             |
| TRIS-HCl                            | 7.0             | 9.0             |
| HEPPSO-NaOH                         | 7.4             | 8.4             |
| BICINE-NaOH-NaCl                    | 7.4             | 9.3             |
| TAPS-NaOH-NaCl                      | 7.45            | 9.35            |
| HEPPS (EPPS)-NaOH                   | 7.5             | 8.7             |
| TRICINE-NaOH                        | 7.6             | 8.6             |
| BICINE-NaOH                         | 7.7             | 8.9             |

Potassium-containing buffers that may be used  
5 in a diagnostic assay according to the present  
invention can be created by adding potassium to the  
buffers shown in the table above that do not contain  
potassium. Potassium-containing buffers useful in a  
diagnostic assay according to the present preferably  
have a K<sup>+</sup> concentration in the range of approximately  
10 2mM to 7mM, more preferably have a K<sup>+</sup> concentration  
of approximately 5mM, and still more preferably have  
a K<sup>+</sup> concentration of 5mM.

The K<sup>+</sup>-containing buffer used in the examples  
15 set forth below is a HEPES buffer to which potassium  
has also been added (5 mM KCl, 4 mM NaHCO<sub>3</sub>, 5 mM  
HEPES, 134 mM NaCl, 2.3 mM CaCl<sub>2</sub>, and 5 mM glucose;  
pH 7.3 - 7.5, preferably 7.4), and is also referred  
to as "regular" or "stock" buffer. The K<sup>+</sup>-free

buffer used in the examples is a HEPES buffer without potassium (4 mM NaHCO<sub>3</sub>, 5 mM HEPES, 134 mM NaCl, 2.3 mM CaCl<sub>2</sub>, and 5 mM glucose; pH 6.6 - 7.0, preferably 7.8).

5       The membrane potential of a patient's cells may be ascertained by any conventional method, such as by examining the fluorescence intensity of a potential-sensitive lipophilic fluorescent dye. The membrane potential is directly proportional to the  
10 intensity of fluorescence according to the following equation:  $I = CV$ , wherein I is the fluorescence intensity of a lipophilic fluorescent dye, V is the voltage or membrane potential, and C is a constant that can vary depending on a number of factors such as, but not limited to, temperature, lamp intensity, number of cells, concentration of the fluorescent dye, incubation time, and lipid composition of cells used. The calibration and determination of the value for C can be a cumbersome and unreliable procedure.  
15 Thus, according to the present invention, by using the ratio of the fluorescence intensity ( $I_1$ ) of one sample of cells to the fluorescence intensity ( $I_2$ ) of another sample of cells, the constant (C) is canceled out. Such ratio-metric measurements are  
20 preferred over absolute measurements.  
25

Examples of potential-sensitive dyes that may be adapted for use in a diagnostic assay according to the present invention, along with their charges and optical responses, are shown below in Table 3  
30 (all available from Molecular Probes Inc., Eugene, OR, US).

TABLE 3

| <u>Dye</u>  | <u>Structure<br/>(Charge)</u> | <u>Optical Response</u>   |
|---|-------------------------------|---|
| DiOC <sub>2</sub> (3)<br>DiOC <sub>5</sub> (3)<br>DiOC <sub>6</sub> (3)<br>DiSC <sub>3</sub> (5)<br>DiIC <sub>1</sub> (5) | Carbocyanine<br>(cationic)    | Slow; fluorescence response to depolarization depends on staining concentration and detection method. |
| JC-1<br>JC-9  | Carbocyanine<br>(cationic)    | Slow; fluorescence emission ratio 585/520 nm increases upon membrane hyperpolarization.               |
| Tetramethyl-<br>rhodamine<br>methyl and<br>ethyl esters<br>Rhodamine 123  | Rhodamine<br>(cationic)       | Slow; used to obtain unbiased images of potential-dependent dye distribution.                         |
| Oxonol V<br>Oxonol VI   | Oxonol<br>(anionic)           | Slow; fluorescence decreases upon membrane hyperpolarization.   |
| DiBAC <sub>4</sub> (3)<br>DiBAC <sub>4</sub> (5)<br>DiSBAC <sub>2</sub> (3)   | Oxonol<br>(anionic)           | Slow; fluorescence decreases upon membrane hyperpolarization.   |
| Merocyanine 540   | Merocyanine                   | Fast/Slow (biphasic response).  |

Indo- (DiI), thia- (DiS) and oxa- (DiO) carbocyanines with short alkyl tails (<7 carbon atoms) were among the first potentiometric fluorescent probes developed. These cationic dyes accumulate on hyperpolarized membranes and are translocated into the lipid bilayer. DiOC<sub>6</sub>(3) (3,3'-dihexyloxacarbocyanine iodide), a cell-permeant, voltage sensitive, green-fluorescent dye, has been the most widely used carbocyanine dye for membrane potential measurements, followed closely by DiOC<sub>5</sub>(3). Thus, in a preferred embodiment of a diagnostic assay according to the present invention, membrane

potentials may be measured using DiOC<sub>6</sub>(3) in conjunction with a fluorescence spectrometer.

One embodiment of the present invention involves a direct comparison of membrane potentials between cells of a patient in need of a bipolar disorder diagnosis and control cells. See, e.g., Example 2 and Figure 2. In particular, this embodiment provides a method for diagnosing a bipolar disorder in a patient, comprising:

10 (a) ascertaining the mean membrane potential of cells of the patient; and one or both of the following steps (b) and (c):

15 (b) comparing the mean membrane potential of the cells of the patient with the mean membrane potential of corresponding control cells of one or more people known to not have said bipolar disorder, wherein a significant difference between the mean membrane potential of the cells of the patient and the mean membrane potential of corresponding control 20 cells indicates that the patient has said bipolar disorder;

25 (c) comparing the mean membrane potential of the cells of the patient with the mean membrane potential of corresponding bipolar control cells of one or more people known to have said bipolar disorder, wherein the lack of a significant difference between the mean membrane potential of the cells of the patient and the mean membrane potential of the corresponding bipolar control cells 30 indicates that the patient has said bipolar disorder.

In one embodiment, steps (a) and (b) are performed. In another embodiment, steps (a) and (c)

are performed. In another embodiment, steps (a), (b) and (c) are performed.

A preferred embodiment of the present invention involves a comparison of the ratios of membrane potentials in K<sup>+</sup>-free buffer to those in K<sup>+</sup>-containing buffer. See, for example, Example 3 and Figure 4. In particular, this embodiment provides a method for diagnosing a bipolar disorder in a patient, comprising:

(a) obtaining a patient ratio of (i) the mean membrane potential of cells of the patient incubated in the absence of K<sup>+</sup> to (ii) the mean membrane potential of cells of the patient incubated in the presence of K<sup>+</sup>; and one or both of the following steps (b) and (c):

(b) comparing the patient ratio obtained in (a) to a control ratio, wherein the control ratio is the ratio of (iii) the mean membrane potential of corresponding control cells of one or more people known to not have said bipolar disorder incubated in the absence of K<sup>+</sup> to (iv) the mean membrane potential of corresponding control cells of one or more people known to not have said bipolar disorder incubated in the presence of K<sup>+</sup>, wherein a significant difference between the patient ratio compared to the control ratio indicates that the patient has said bipolar disorder;

(c) comparing the patient ratio obtained in (a) to a bipolar control ratio, wherein the bipolar control ratio is the ratio of (v) the mean membrane potential of corresponding bipolar control cells of one or more people known to have said bipolar disorder incubated in the absence of K<sup>+</sup> to (vi) the

mean membrane potential of corresponding bipolar control cells of one or more people known to have | said bipolar disorder incubated in the presence of K<sup>+</sup>, wherein the lack of a significant difference 5 between the patient ratio and the bipolar control | ratio indicates that the patient has said bipolar disorder.

In one embodiment, steps (a) and (b) are performed. In another embodiment, steps (a) and (c) 10 are performed. In another embodiment, steps (a), (b) and (c) are performed.

In a preferred embodiment, the significant difference between the patient ratio compared to the control ratio is that the patient ratio is 15 significantly higher than the control ratio.

These comparative ratios, wherein the patient ratio is compared to the control ratio and/or the bipolar control ratio, can be illustrated as follows:

patient ratio:

(i) mean membrane potential of cells of the patient incubated in the absence of K<sup>+</sup>

5 (ii) mean membrane potential of cells of the patient incubated in the presence of K<sup>+</sup>

compared to:

10 control ratio:

(iii) mean membrane potential of corresponding control cells incubated in the absence of K<sup>+</sup>

15 (iv) mean membrane potential of corresponding control cells incubated in the presence of K<sup>+</sup>

and/or compared to:

20 bipolar control ratio:

(v) mean membrane potential of corresponding bipolar control cells incubated in the absence of K<sup>+</sup>

25 (vi) mean membrane potential of corresponding bipolar control cells incubated in the presence of K<sup>+</sup>.

30 According to the above, when K<sup>+</sup> is present, it is preferably present at a concentration of approximately 2 - 7 mM, more preferably at a concentration of approximately 5mM, and still more preferably at a concentration of 5mM.

35 In another embodiment, the cells of the patient are incubated in the presence of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity; and the corresponding control cells, the corresponding bipolar control cells, or both the corresponding control cells and the corresponding bipolar control cells are also 40 incubated in the presence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity.

yet another preferred embodiment of the present invention involves a comparison of the ratio of membrane potential with and without a  $\text{Na}^+ \text{K}^+$  ATPase-altering compound. See, e.g., Example 5 and Figure 5 8, and Example 10 and Figure 15. In particular, this | embodiment provides a method for diagnosing a bipolar disorder in a patient, comprising:

(a) obtaining a patient ratio of (i) the mean membrane potential of cells of the patient incubated 10 in the presence of a compound that alters  $\text{Na}^+ \text{K}^+$  ATPase activity to (ii) the mean membrane potential of cells of the patient incubated in the absence of the compound that alters  $\text{Na}^+ \text{K}^+$  ATPase activity; and one or both of the following steps (b) and (c):

15 (b) comparing the patient ratio obtained in (a) to a control ratio, wherein the control ratio is the ratio of (iii) the mean membrane potential of corresponding control cells of one or more people known to not have said bipolar disorder incubated in 20 the presence of a compound that alters  $\text{Na}^+ \text{K}^+$  ATPase activity to (iv) the mean membrane potential of corresponding control cells of one or more people known to not have said bipolar disorder incubated in 25 the absence of the compound that alters  $\text{Na}^+ \text{K}^+$  ATPase activity, wherein a significantly lower patient ratio compared to the control ratio indicates that the patient has said bipolar disorder;

(c) comparing the patient ratio obtained in (a) to a bipolar control ratio, wherein the bipolar 30 control ratio is the ratio of (v) the mean membrane potential of corresponding bipolar control cells of one or more people known to have said bipolar disorder incubated in the presence of a compound

that alters  $\text{Na}^+/\text{K}^+$  ATPase activity to (vi) the mean membrane potential of corresponding bipolar control cells of one or more people known to have said bipolar disorder incubated in the absence of the compound that alters  $\text{Na}^+/\text{K}^+$  ATPase activity, wherein the lack of a significant difference between the patient ratio compared to the bipolar control ratio indicates that the patient has said bipolar disorder.

In one embodiment, steps (a) and (b) are performed. In another embodiment, steps (a) and (c) are performed. In another embodiment, steps (a), (b) and (c) are performed.

These comparative ratios, wherein the patient ratio is compared to the control ratio and/or the bipolar control ratio, can be illustrated as follows:

patient ratio:

(i) mean membrane potential of cells of the patient incubated in the presence of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity

5

(ii) mean membrane potential of cells of the patient incubated in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity

10 compared to:

control ratio:

(iii) mean membrane potential of corresponding control cells incubated in the presence of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity

15

(iv) mean membrane potential of corresponding control cells incubated in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity

20

and/or compared to:

bipolar control ratio:

(v) mean membrane potential of corresponding bipolar control cells incubated in the presence of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity

25

(vi) mean membrane potential of corresponding bipolar control cells incubated in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity.

30

In one embodiment, the cells are incubated in the presence of K<sup>+</sup>, wherein the K<sup>+</sup> concentration is preferably approximately 2 - 7 mM, more preferably approximately 5mM, and still more preferably 5mM. In another embodiment, the cells are incubated in the absence of K<sup>+</sup>. Preferably, the cells incubated in the presence of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity are incubated in the absence of K<sup>+</sup>, and the

40

cells incubated in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity are incubated in the presence of K<sup>+</sup>.

Still another preferred embodiment of the present invention involves a comparison of the relative ratios of membrane potentials in K<sup>+</sup>-free buffer to those in K<sup>+</sup>-containing buffer with and without a Na<sup>+</sup>K<sup>+</sup> ATPase-altering compound. See, e.g., Examples 4 and 7 and Figures 5 and 11. In particular, this embodiment provides a method for diagnosing a bipolar disorder in a patient, comprising:

(a) obtaining a ratio (patient ratio I) of (i) the mean membrane potential of cells of the patient incubated in the absence of K<sup>+</sup> and in the presence of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity to (ii) the mean membrane potential of cells of the patient incubated in the absence of K<sup>+</sup> and in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity;

(b) obtaining a ratio (patient ratio II) of (iii) the mean membrane potential of cells of the patient incubated in the presence of K<sup>+</sup> and in the presence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity to (iv) the mean membrane potential of cells of the patient incubated in the presence of K<sup>+</sup> and in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity;

(c) obtaining a relative ratio (Relative Patient Ratio) of patient ratio I to patient ratio II; and one or both of the following steps (d) and (e):

(d) comparing the Relative Patient Ratio to a Relative Control Ratio, wherein the Relative Control Ratio is the relative ratio of control ratio I to

control ratio II, wherein control ratio I is the ratio of (i') the mean membrane potential of corresponding control cells of one or more people known to not have said bipolar disorder incubated in the absence of K<sup>+</sup> and in the presence of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity to (ii') the mean membrane potential of corresponding control cells of one or more people known to not have said bipolar disorder incubated in the absence of K<sup>+</sup> and in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity, and wherein control ratio II is the ratio of (iii') the mean membrane potential of corresponding control cells of one or more people known to not have said bipolar disorder incubated in the presence of K<sup>+</sup> and in the presence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity to (iv') the mean membrane potential of corresponding control cells of one or more people known to not have said bipolar disorder incubated in the presence of K<sup>+</sup> and in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity, wherein a significant difference between the Relative Patient Ratio compared to the Relative Control Ratio indicates that the patient has said bipolar disorder;

(e) comparing the Relative Patient Ratio to a Relative Bipolar Control Ratio, wherein the Relative Bipolar Control Ratio is the relative ratio of bipolar control ratio I to bipolar control ratio II, wherein bipolar control ratio I is the ratio of (i'') the mean membrane potential of corresponding bipolar control cells of one or more people known to have said bipolar disorder incubated in the absence of K<sup>+</sup> and in the presence of a compound that alters

Na<sup>+</sup>K<sup>+</sup> ATPase activity to (ii'') the mean membrane potential of corresponding bipolar control cells of one or more people known to have said bipolar disorder incubated in the absence of K<sup>+</sup> and in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity, and wherein bipolar control ratio II is the ratio of (iii'') the mean membrane potential of corresponding bipolar control cells of one or more people known to have said bipolar disorder incubated in the presence of K<sup>+</sup> and in the presence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity to (iv'') the mean membrane potential of corresponding bipolar control cells of one or more people known to have said bipolar disorder incubated in the presence of K<sup>+</sup> and in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity, wherein the lack of a significant difference between the Relative Patient Ratio compared to the Relative Bipolar Control Ratio indicates that the patient has bipolar disorder.

In one embodiment, steps (a), (b), (c), and (d) are performed. In another embodiment, steps (a), (b), (c), and (e) are performed. In another embodiment, steps (a), (b), (c), (d), and (e) are performed.

In a preferred embodiment, the significant difference between the Relative Patient Ratio and the Relative Control Ratio is that the Relative Patient Ratio is significantly higher than the Relative Control Ratio.

These comparative ratios, wherein the Relative Patient Ratio is compared to the Relative Control Ratio and/or the Relative Bipolar Control Ratio, can be illustrated as follows:

**Relative Patient Ratio:**

patient ratio I:

5 (i) mean membrane potential of cells of the patient  
incubated in the absence of K<sup>+</sup> and in the presence  
of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity

---

10 (ii) mean membrane potential of cells of the patient  
incubated in the absence of K<sup>+</sup> and in the absence of  
the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity

---

15 patient ratio II:

(iii) mean membrane potential of cells of the patient  
incubated in the presence of K<sup>+</sup> and in the  
presence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase  
activity

---

20 (iv) mean membrane potential of cells of the patient  
incubated in the presence of K<sup>+</sup> and in the absence  
of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity

---

**compared to:**

**Relative Control Ratio:**

25 control ratio I:

(i') mean membrane potential of corresponding  
control cells incubated in the absence of K<sup>+</sup> and in  
the presence of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase  
activity

---

30 (ii') mean membrane potential of corresponding  
control cells incubated in the absence of K<sup>+</sup> and in  
the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase  
activity

---

35 control ratio II:

40 (iii') mean membrane potential of corresponding  
control cells incubated in the presence of K<sup>+</sup> and in  
the presence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase  
activity

---

45 (iv') mean membrane potential of corresponding  
control cells incubated in the presence of K<sup>+</sup> and in  
the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase  
activity

and/or compared to:

**Relative Bipolar Control Ratio:**

bipolar control ratio I:

5 (i'') mean membrane potential of corresponding bipolar control cells incubated in the absence of K<sup>+</sup> and in the presence of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity

10 (ii'') mean membrane potential of corresponding bipolar control cells incubated in the absence of K<sup>+</sup> and in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity

15 bipolar control ratio II:

20 (iii'') mean membrane potential of corresponding bipolar control cells incubated in the presence of K<sup>+</sup> and in the presence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity

25 (iv'') mean membrane potential of corresponding bipolar control cells incubated in the presence of K<sup>+</sup> and in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity.

Yet another embodiment of the present invention involves a comparison of the repolarization rate 30 between cells of a patient being diagnosed and control cells. See, e.g., Example 4 and Figure 7. In particular, this embodiment provides a method for diagnosing a bipolar disorder in a patient, comprising:

35 (a) ascertaining a mean rate of repolarization in cells of the patient incubated in the presence of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity; and one or both of the following steps (b) and (c):

40 (b) comparing the mean rate of repolarization ascertained in (a) to the mean rate of repolarization in corresponding control cells of one

or more people known to not have said bipolar disorder incubated in the presence of the compound that alters  $\text{Na}^+ \text{K}^+$  ATPase activity, wherein a significant difference between the mean rate of repolarization in the cells of the patient compared to the mean rate of repolarization in the corresponding control cells indicates that the patient has said bipolar disorder;

(c) comparing the mean rate of repolarization ascertained in (a) to the mean rate of repolarization in corresponding bipolar control cells of one or more people known to have said bipolar disorder incubated in the presence of the compound that alters  $\text{Na}^+ \text{K}^+$  ATPase activity, wherein the lack of a significant difference between the mean rate of repolarization in the cells of the patient compared to the mean rate of repolarization in the corresponding bipolar control cells indicates that the patient has said bipolar disorder.

In one embodiment, steps (a) and (b) are performed. In another embodiment, steps (a) and (c) are performed. In another embodiment, steps (a), (b) and (c) are performed.

In a preferred embodiment, the significant difference between the mean rate of repolarization in the cells of the patient compared to the mean rate of repolarization in the corresponding control cells is that the mean rate of repolarization in the cells of the patient is significantly higher (e.g.  $P < 0.05$ ) than the mean rate of repolarization in the corresponding control cells.

In one embodiment, the cells are incubated in the presence of  $\text{K}^+$ . In another embodiment, the cells

are incubated in the absence of K<sup>+</sup>. As used herein, "presence of K<sup>+</sup>" preferably means a K<sup>+</sup> concentration in the range of approximately 2mM to 7mM, preferably approximately 5mM. For example, the K<sup>+</sup>-containing HEPES buffer used in the examples below has a K<sup>+</sup> concentration of 5mM.

In yet another embodiment, the present invention provides a method for diagnosing unipolar disorder in a patient, comprising:

(a) obtaining a patient ratio of (i) the mean membrane potential of cells of the patient incubated in the presence of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity to (ii) the mean membrane potential of cells of the patient incubated in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity; and one or both of the following steps (b) and (c):

(b) comparing the patient ratio obtained in (a) to a control ratio, wherein the control ratio is the ratio of (iii) the mean membrane potential of corresponding control cells of one or more people known to not have unipolar disorder incubated in the presence of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity to (iv) the mean membrane potential of corresponding control cells of one or more people known to not have unipolar disorder incubated in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity, wherein a significantly higher patient ratio compared to the control ratio indicates that the patient has unipolar disorder;

(c) comparing the patient ratio obtained in (a) to a unipolar control ratio, wherein the unipolar control ratio is the ratio of (v) the mean membrane potential of corresponding unipolar control

cells of one or more people known to have unipolar disorder incubated in the presence of a compound that alters  $\text{Na}^+ \text{K}^+$  ATPase activity to (vi) the mean membrane potential of corresponding unipolar control 5 cells of one or more people known to have unipolar disorder incubated in the absence of the compound that alters  $\text{Na}^+ \text{K}^+$  ATPase activity, wherein the lack of a significant difference between the patient ratio compared to the unipolar control ratio 10 indicates that the patient has unipolar disorder.

In one embodiment, steps (a) and (b) are performed. In another embodiment, steps (a) and (c) are performed. In another embodiment, steps (a), (b) and (c) are performed.

15 These comparative ratios, wherein the patient ratio is compared to the control ratio and/or the unipolar control ratio, can be illustrated as follows:

patient ratio:

(i) mean membrane potential of cells of the patient incubated in the presence of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity

5

(ii) mean membrane potential of cells of the patient incubated in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity

10 compared to:

control ratio:

(iii) mean membrane potential of corresponding control cells incubated in the presence of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity

15

(iv) mean membrane potential of corresponding control cells incubated in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity

20

and/or compared to:

unipolar control ratio:

(v) mean membrane potential of corresponding unipolar control cells incubated in the presence of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity

25

(vi) mean membrane potential of corresponding unipolar control cells incubated in the absence of the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity.

30

In one embodiment, the cells are incubated in the presence of K<sup>+</sup>, wherein the K<sup>+</sup> concentration is preferably approximately 2 - 7 mM, more preferably approximately 5mM, and still more preferably 5mM. In another embodiment, the cells are incubated in the absence of K<sup>+</sup>. Preferably, the cells incubated in the presence of a compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase activity are incubated in the absence of K<sup>+</sup>, and the

40

cells incubated in the absence of the compound that alters  $\text{Na}^+\text{K}^+$  ATPase activity are incubated in the presence of  $\text{K}^+$ .

In a preferred embodiment of a diagnostic assay for a bipolar disorder or unipolar disorder according to the present invention, the compound that alters  $\text{Na}^+\text{K}^+$  ATPase activity is a compound selected from the group consisting of: valinomycin, monensin, gramicidin, *p*-chloromercurybenzenesulfonate (PCMBS), monensin decyl ester, veratridine, ethacrynat, dopamine, a catecholamine, a phorbol ester, ouabain, lithium, valproate, lamotrigine, cocaine, nicotine, the protein kinase C inhibitor R0-31-8220 (Wilkinson et al, *Biochem. J.* 294:335-337 (1993)), oxymetazoline, calcineurin, topiramate, a peptide hormone, sorbitol, and a diuretic. Preferably, the compound that alters  $\text{Na}^+\text{K}^+$  ATPase activity increases  $\text{Na}^+\text{K}^+$  ATPase activity.

In one embodiment of a diagnostic assay for a bipolar disorder or a diagnostic assay for unipolar disorder according to the present invention, the compound that alters  $\text{Na}^+\text{K}^+$  ATPase activity is ethacrynat. Preferably, the concentration of ethacrynat is in the range of 1  $\mu\text{M}$  to 100  $\mu\text{M}$ , for example approximately 30  $\mu\text{M}$ , more preferably 30  $\mu\text{M}$ .

In another embodiment of a diagnostic assay for a bipolar disorder or a diagnostic assay for unipolar disorder according to the present invention, the compound that alters  $\text{Na}^+\text{K}^+$  ATPase activity is monensin. Preferably, the concentration of monensin is in the range of 1  $\mu\text{M}$  to 50  $\mu\text{M}$ , for example approximately 10  $\mu\text{M}$ , more preferably 10  $\mu\text{M}$ .

In another embodiment of a diagnostic assay for  
| a bipolar disorder or a diagnostic assay for  
unipolar disorder according to the present  
invention, the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase  
5 activity is monensin decyl ester. Preferably, the  
concentration of monensin decyl ester is in the  
range of 1 μM to 50 μM, for example approximately 10  
μM, more preferably 10 μM.

In another embodiment of a diagnostic assay for  
10 | a bipolar disorder or a diagnostic assay for  
unipolar disorder according to the present  
invention, the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase  
activity is a phorbol ester. Preferably, the phorbol  
ester is selected from the group consisting of:  
15 phorbol 12-myristate 13-acetate (PMA), 12-O-  
tetradecanoylphorbol 13-acetate, phorbol 12-  
myristate 13-acetate 4-O-methyl ether, phorbol 12,  
13-dibutyrate (PDBu), phorbol 12,13-didecanoate  
(PDD), and phorbol 12,13-dinonanoate 20-  
20 homovanillate. More preferably, the phorbol ester  
is phorbol 12-myristate 13-acetate (PMA).  
Preferably, the concentration of phorbol 12-  
myristate 13-acetate (PMA) is in the range of 0.1 μM  
to 10 μM, for example approximately 2 μM, more  
25 preferably 2 μM.

In another embodiment of a diagnostic assay for  
| a bipolar disorder or a diagnostic assay for  
unipolar disorder according to the present  
invention, the compound that alters Na<sup>+</sup>K<sup>+</sup> ATPase  
30 activity is lithium. Preferably, the concentration  
of lithium is in the range of 1 μM to 50 μM, for  
example approximately 20 mM, more preferably 20 mM.

In another preferred embodiment of a diagnostic assay for a bipolar disorder or a diagnostic assay for unipolar disorder according to the present invention, the mean membrane potential of the cells of the patient is determined by incubating the cells of the patient with a voltage-sensitive fluorescent dye and measuring the fluorescence intensity of the fluorescent dye. Preferably, the dye is a cell-permeant cationic dye. Preferably, the dye is a carbocyanine dye. In a preferred embodiment, the dye is 3,3'-dihexyloxacarbocyanine iodide DiOC<sub>6</sub>(3). Preferably, the concentration of dye is in the range of 1 nM to 500 nM, for example approximately 7 nM, more preferably 7 nM.

In another preferred embodiment of a diagnostic assay for a bipolar disorder or a diagnostic assay for unipolar disorder according to the present invention, the cells of the patient and the corresponding control cells (and corresponding bipolar and unipolar control cells, as appropriate) are selected from the group consisting of: lymphoblasts, erythrocytes, platelets, leukocytes, macrophages, monocytes, dendritic cells, fibroblasts, epidermal cells, mucosal tissue cells, cells in the cerebrospinal fluid, hair cells, and cells in whole blood. Preferably, the cells are lymphoblasts or cells in whole blood.

As described more fully in the examples below, the ratio of membrane potentials of bipolar cells in K<sup>+</sup>-free buffer to membrane potentials of bipolar cells in K<sup>+</sup>-containing buffer is significantly different from that of unaffected cells. Cells incubated in ethacrynatate (30μM) show significant

differences between bipolar cells and unaffected cells. Cells incubated in monensin ( $10\mu M$ ) and in PMA ( $2\mu M$ ) also show significant differences in specific potentials. In addition to being a mood stabilizer,  
5 lithium serves as a medium to distinguish between bipolar cells and unaffected cells.

Further, as shown in a clinical trial using whole blood samples from bipolar, unipolar and schizophrenic patients described more fully below,  
10 the ratio of membrane potentials of bipolar cells is significantly different from that of matched control cells ( $P < 0.001$ ). In addition, the ratio of membrane potentials of unipolar cells is significantly different from that of matched control  
15 cells ( $P < 0.001$ ).

According to the present invention, when two sample groups are compared for differences for diagnostic purposes, e.g. affected cells and unaffected cells, student's t-test is preferably employed to determine if the two groups are significantly different from each other. As used herein, a "significant difference" (i.e., significantly higher or significantly lower) means that  $P < 0.05$ . Commercially available statistical  
25 software is preferably used for this purpose.

According to the present invention, when more than two sample groups are compared to diagnose whether or not an individual patient is bipolar or unipolar, analysis of variance (ANOVA) test is  
30 preferably used. (See Beth Dawson and Robert G. Trapp, Basic and Clinical Biostatistics, Lange Medical Books, McGraw-Hill 3rd Edn.) In a preferred embodiment, an individual patient's cell sample is

tested as described herein and six to twelve values are calculated. These patient values are treated as a first sample group and compared with a normal control group (negative control) and a bipolar group 5 (positive control) as shown in Example 10. A bipolar diagnosis is made when there is a significant difference ( $P < 0.05$ ) between the normal control group (negative control) and the patient, but there is not a significant difference between the bipolar 10 control group (positive control) and the patient. A similar procedure is used to determine whether or not a patient is unipolar.

Although the examples below describe experiments comparing membrane potentials of 15 patients being diagnosed for a bipolar disorder to groups of controls that were examined contemporaneously, an assay according to the invention preferably compares the mean membrane potential of a patient's cells to predetermined 20 control value or values. Thus, the standard control value(s) do not need to be determined contemporaneously with every patient assay for a bipolar disorder, but are preferably pre-determined for later comparisons with patients being diagnosed. 25 Although not contemporaneous, assay conditions between patients and controls are preferably the same. Preferably, control data is pre-determined from various types of corresponding control cells, so that regardless of the type of cells from a 30 patient being tested, control information already exists with which to make a comparison and thus a diagnosis. Furthermore, control data is preferably pre-determined using a control group known not to

have any mental illness, in addition to control groups having mental illnesses such as a bipolar disorder, unipolar disorder, or schizophrenia.

The instant disclosure demonstrates that manipulation of  $\text{Na}^+ \text{K}^+$  ATPase activity provides an effective tool for differentiating bipolar cells from non-bipolar cells. Novel diagnostic assays for a bipolar disorder are thus provided. The instant disclosure also demonstrates that manipulation of  $\text{Na}^+ \text{K}^+$  ATPase activity is an effective tool for differentiating unipolar cells from non-unipolar cells, and therefore provides diagnostic assays for unipolar disorder. The specificity and sensitivity of the diagnostic assays described herein compare well with state-of-the-art diagnostic techniques for various other diseases.

The following examples are provided for illustrative purposes only and are in no way intended to limit the scope of the invention.

ExamplesExample 1Cell Cultures

5        Three groups of immortalized lymphoblast samples were obtained from the Human Genetic Mutant Cell Repository (Coriell Institute for Medical Research, Camden NJ). The samples are shown in Table 4 below and included six from affected  
 10 | (bipolar I disorder) patients (A1 through A6), six from their siblings (S1 through S6), and six from unrelated normal control subjects matched by age and sex (N1 through N6).

TABLE 4

| Name | Coriell Number | Age   | Sex    |
|------|----------------|-------|--------|
| A1   | GM05977GM09869 | 26 yr | Male   |
| A2   | GM05999GM05977 | 22 yr | Female |
| A3   | GM05918GM05999 | 26 yr | Female |
| A4   | GM06003GM05918 | 16 yr | Female |
| A5   | GM11051GM06003 | 35 yr | Female |
| A6   | GM05933GM11051 | 25 yr | Female |
| S1   | GM05914GM05933 | 23 yr | Male   |
| S2   | GM05901GM05914 | 28 yr | Female |
| S3   | GM09215GM05901 | 50 yr | Male   |
| S4   | GM05888GM09215 | 18 yr | Male   |
| S5   | GM05901GM05888 | 28 yr | Female |
| S6   | GM05945GM05901 | 55 yr | Female |
| N1   | GM06160GM05945 | 25 yr | Male   |
| N2   | GM05408GM06160 | 28 yr | Female |
| N3   | GM06862GM05408 | 34 yr | Female |
| N4   | GM06051GM06862 | 26 yr | Female |
| N5   | GM06861GM06051 | 36 yr | Male   |
| N6   | GM09869GM06861 | 36 yr | Male   |

15       The cells were grown at 37°C in RPMI 1640 culture medium (GIBCO, Life Technologies, Gaithersburg, MD, USA) with 15% fetal bovine serum and 1% penicillin.

**Example 2**Determination of Membrane Potential

The membrane potentials of the bipolar I cells shown in Table 4 above (as well as in all other experiments, except where indicated) were measured using the following protocol. The cells cultured in the media described above were centrifuged at 210g for 5 minutes at room temperature, then suspended in 3 ml of K<sup>+</sup>-containing stock buffer (5 mM KCl, 4 mM NaHCO<sub>3</sub>, 5 mM HEPES, 134 mM NaCl, 2.3 mM CaCl<sub>2</sub>, and 5 mM glucose). The cells were counted and the desired number of cells were again suspended in the K<sup>+</sup>-containing buffer. The membrane potentials were measured using 3,3'-dihexyloxacarbocyanine iodide DiOC<sub>6</sub>(3), a cell-permeant, voltage sensitive, green-fluorescent dye (Molecular Probes Inc. Eugene, OR USA). The cells were preincubated for 30 minutes before measurements. The preincubated cell suspension was centrifuged at 210 g for 5 minutes and the cells were resuspended in the K<sup>+</sup>-containing buffer without the dye in the buffer.

A fluorescence spectrometer (F2500 Hitachi, Japan) was used for the measurement of the membrane potential by measuring the fluorescence intensity of DiOC<sub>6</sub>(3), which is directly proportional to the membrane potential. The intensity of fluorescence was measured at an excitation wavelength of 488 nm and an output wavelength of 540 nm. The time of recordings varied from 10 seconds to 1500 seconds depending upon the experiment.

Membrane potential was expressed according to fluorescence intensity using the following equation:  
I = CV, wherein I is the fluorescence intensity of a

lipophilic fluorescent dye DiOC<sub>6</sub>(3) being only one example of such a dye); V is the voltage or membrane potential; and C is a constant that can vary depending on a number of factors such as, but not limited to, temperature, lamp intensity, number of cells, concentration of the fluorescent dye, incubation time, and lipid composition of cells used. By using the ratio of the fluorescence intensity ( $I_1$ ) of one sample of cells to the fluorescence intensity ( $I_2$ ) of another sample of cells, the constant (C) could be canceled out

The measurements of the fluorescence intensity (and thereby membrane potential, per the above equation) indicated that the mean membrane potential of the affected (bipolar\_I) cells was statistically distinguishable from that of the other cells. Figure 1 shows box plots of the membrane potentials of lymphoblasts from the three groups tested, namely the affected (bipolar\_I) cells, non-affected sibling cells, and normal cells, as indicated by the fluorescence intensity of DiOC<sub>6</sub>(3) (7 nM) in K<sup>+</sup>-containing (regular) buffer. The differences in the mean values among the groups were greater than would be expected by chance. The mean fluorescence intensity of the affected (bipolar\_I) cells was significantly lower ( $P < 0.001$ ) by a one-way ANOVA test than that of the sibling cells as well as that of the normal control cells. There was no significant difference between normal cells and sibling cells. The box plots show the 5<sup>th</sup>, 25<sup>th</sup>, 50<sup>th</sup>, 75<sup>th</sup>, and 95<sup>th</sup> percentile lines. The mean and median lines coincide for the affected cells, but are separated for the other two cell lines. Power of

performed test with alpha = 0.050: 0.991. N = 6.

Data points = 57.

A post-hoc comparison showed a significant difference between affected (bipolar\_I) cells vs. normal cells and sibling cells, but not between sibling cells and normal cells. Therefore sibling cells and normal cells were combined into a single, "unaffected" category. Figure 2 shows the membrane potentials of affected (bipolar\_I) cells and unaffected cells, as indicated by the fluorescence intensity of DiOC<sub>6</sub>(3) in affected cells and in the combination of non-affected sibling cells and normal cells. The difference in the mean values of the resulting two groups was greater than would be expected by chance. The mean fluorescence intensity of the affected (bipolar\_I) cells was significantly lower ( $P < 0.001$ ) than that of the unaffected cells. 95 percent confidence interval for difference of means: -159.577 to -66.090. Power of performed test with alpha = 0.050: 0.999.

An additional ratio-metric comparison was made by plotting a ratio of fluorescence intensities of the tested cells to that of a cell selected as a standard. Figure 3 shows the fluorescence intensity of DiOC<sub>6</sub>(3) in affected (bipolar\_I) cells and unaffected (sibling and normal) cells relative to the fluorescence intensity of DiOC<sub>6</sub>(3) in the control cell, N1. This ratio-metric procedure accounted for variations in cell count and dye concentration. The difference in the mean values of the resulting two groups was greater than would be expected by chance. The mean relative intensity of the affected (bipolar\_I) cells was significantly lower ( $P < 0.001$ ) than

that of the unaffected cells.  $t = -5.386$  with 54 degrees of freedom. Power of performed test with alpha = 0.050: 1.000.

The data presented in this example and shown in  
5 Figures 1-3 indicates that the membrane potential of  
cells such as lymphoblasts can be used to  
distinguish groups of patients with bipolar I  
disorder from groups of unaffected controls.

10

### Example 3

#### Membrane Potential In K<sup>+</sup>-free Buffer

Membrane potentials were also measured in K<sup>+</sup>-free buffer to enable comparison with membrane potentials in K<sup>+</sup>-containing buffer. As used herein,  
15 K<sup>+</sup>-free buffer contains all of the components of the K<sup>+</sup>-containing buffer, except potassium (4 mM NaHCO<sub>3</sub>, 5 mM HEPES, 134 mM NaCl, 2.3 mM CaCl<sub>2</sub>, and 5 mM glucose). Constant volumes of samples of each cell line were added to an equal volume of buffer  
20 containing an equal volume of dye. K<sup>+</sup>-free buffer as well as K<sup>+</sup>-containing buffer contained an equal volume of cells (and hence an equal number of cells from that culture sample) and an equal concentration of dye. Each variable was constant for each cell  
25 line in both buffers, so that the ratio of intensities was a true ratio-metric measurement.

Figure 4 shows a comparison of membrane potentials in K<sup>+</sup>-free buffer with those in K<sup>+</sup>-containing (regular) buffer, as indicated by  
30 fluorescence intensity. 70 nM DiOC<sub>6</sub>(3) was added to the cells 30 minutes prior to measuring the membrane potential. The ratio of fluorescence intensity of DiOC<sub>6</sub>(3) in K<sup>+</sup>-free buffer to the fluorescence

intensity of DiOC<sub>6</sub>(3) in K<sup>+</sup>-containing buffer was plotted in the y-axis. The mean value of the ratio of the membrane potential of affected cells was approximately 0.71. In comparison, the mean value of  
5 the ratio of the membrane potential of the unaffected cells was approximately 0.51. The ratio of the membrane potential of the unaffected cells was significantly lower than that in affected cells, as indicated by t-test (P = 0.020).

10 The above procedure was also used for the ratio-metric measurements described below.

#### **Example 4**

##### Ethacrynone-Induced Changes In Membrane Potential

15 Ethacrynone is a loop diuretic prescribed for kidney patients that increases the intracellular sodium concentration and decreases the intracellular potassium concentration. When the intracellular sodium concentration increases, cells correspondingly regulate Na<sup>+</sup>K<sup>+</sup> ATPase activity to  
20 pump the extra sodium out of the cells. Thus, these changes initiate the Na<sup>+</sup>K<sup>+</sup> ATPase activity to normalize the ionic gradients.

25 The relative fluorescence intensity ratio of cells in K<sup>+</sup>-free buffer was compared to that of cells in K<sup>+</sup>-containing buffer with or without 30 μM ethacrynone. The cells were incubated with or without ethacrynone (30 μM) for 30 min in K<sup>+</sup>-containing buffer or in K<sup>+</sup>-free buffer. The membrane  
30 potential of these cells was determined by measuring the fluorescence intensity as described above. The intensity ratio (membrane potential with ethacrynone/membrane potential without ethacrynone)

was compared to the intensity ratio in K<sup>+</sup>-containing buffer and/or in K<sup>+</sup>-free buffer. The relative intensity ratio was obtained by dividing the intensity ratio in K<sup>+</sup>-free buffer by the intensity ratio in K<sup>+</sup>-containing buffer.

A constant sample volume of each cell line was added to an equal volume of each buffer containing an equal volume of dye. Thus, the K<sup>+</sup>-free buffer and the K<sup>+</sup>-containing buffer contained an equal number of 10 cells from each culture sample and an equal concentration of dye.

As shown in Figure 5, the relative intensity ratio of affected cells was significantly higher than that of unaffected cells by t-test. The mean 15 value of the ratio of the fluorescence intensity of affected cells in K<sup>+</sup>-free buffer to the fluorescence intensity of affected cells in K<sup>+</sup>-containing (regular) buffer was approximately 1.04. The mean value of the ratio of fluorescent intensity of the 20 unaffected cells in K<sup>+</sup>-free buffer to the fluorescence intensity of unaffected cells in K<sup>+</sup>-containing buffer was approximately 0.79. Further, the mean value of the ratio of the affected cells 25 was statistically higher than the mean ratio of unaffected cells ( $P = 0.009$ ).

Similar differences were found in K<sup>+</sup>-containing buffer alone or in K<sup>+</sup>-free buffer alone. The fluorescence intensity of cells incubated in K<sup>+</sup>-free buffer with 30  $\mu$ M ethacrynatate was compared to that 30 of cells incubated in K<sup>+</sup>-free buffer without ethacrynatate. The membrane potential of these cells was determined by measuring the fluorescence intensity as described above. As shown in Figure 6,

the intensity ratio (membrane potential of cells incubated with ethacrynatemembrane potential of cells not incubated with ethacrynatemembrane potential) of affected cells was significantly higher than that of 5 unaffected cells by t-test ( $P = 0.032$ ).

As the  $\text{Na}^+\text{K}^+$  ATPase density on the cell surface increases, the intracellular sodium concentration decreases and the membrane potential begins to drop as a result of repolarization of the cell membrane.  
10 The rate of drop in membrane potential (repolarization rate) in affected cells incubated for 30 minutes in both  $\text{K}^+$ -free buffer and in  $\text{K}^+$ -containing (regular) buffer in the presence or absence of 30  $\mu\text{M}$  ethacrynatemembrane potential was compared to that of 15 unaffected cells incubated for 30 minutes in both  $\text{K}^+$ -free buffer and in  $\text{K}^+$ -containing buffer in the presence or absence of 30  $\mu\text{M}$  ethacrynatemembrane potential. The difference in the median values between the two groups was greater than would be expected by chance.  
20 As shown in Figure 7, the relative rate of repolarization was significantly higher in affected cells compared to that of unaffected cells ( $P = 0.041$ , as determined by t-test).

25

#### **Example 5**

##### Monensin-Induced Changes In Membrane Potential

Monensin is an antibiotic ionophore having a 10-fold preference for  $\text{Na}^+$  over  $\text{K}^+$ . Monensin increases the intracellular sodium concentration,  
30 thus changing the ratio of membrane potentials. The increase in sodium concentration in turn leads to an increase in  $\text{Na}^+\text{K}^+$  ATPase density.

The fluorescence intensity of cells incubated in K<sup>+</sup>-free buffer with 10 μM monensin was compared to that of cells incubated in K<sup>+</sup>-free buffer without monensin. The cells were incubated for 30 minutes.

5      The membrane potential of these cells was determined by measuring the fluorescence intensity as described above. As shown in Figure 8, the intensity ratio (membrane potential of cells incubated with monensin/membrane potential of cells not incubated with monensin) of affected cells was significantly lower than that of unaffected cells by t-test (P = 10      0.009).

#### **Example 6**

15      PMA-Induced Changes In Membrane Potential

Protein kinase C (PKC) is an essential enzyme involved in the phosphorylation of ATP, which provides energy for the Na<sup>+</sup>K<sup>+</sup> ATPase. Furthermore, PKC activators such as PMA and inhibitors such as 20      dopamine significantly alter Na<sup>+</sup>K<sup>+</sup> ATPase density in the presence of monensin.

The rate of drop in membrane potential after incubation with the protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA), 25      was compared in affected cells to that of unaffected cells. The membrane potential of these cells was determined by measuring the fluorescence intensity as described above. The rate of drop in membrane potential (repolarization rate) in affected cells 30      was compared to that in unaffected cells in K<sup>+</sup>-free buffer in the presence or absence of 2 μM PMA. The difference in the median values between the two groups was greater than would be expected by chance.

The ratio of repolarization rate with PMA divided by the repolarization rate without PMA is shown in Figure 9. Figure 9 shows that when the cells were incubated in 2  $\mu$ M PMA before membrane potential measurements, the repolarization rate in affected cells was significantly different from the repolarization rate in unaffected cells by t-test ( $P = 0.029$ ).

Figure 10 shows the effect of PMA on the relative ratio of repolarization rates. The rate of drop in membrane potential (repolarization rate) in affected cells incubated for 30 minutes in both K<sup>+</sup>-free buffer and in K<sup>+</sup>-containing (regular) buffer in the presence or absence of 2  $\mu$ M PMA was compared to that of unaffected cells incubated for 30 minutes in both K<sup>+</sup>-free buffer and in K<sup>+</sup>-containing buffer in the presence or absence of 2  $\mu$ M PMA. The difference in the median values between the two groups was greater than would be expected by chance. As shown in Figure 10, the relative ratio of repolarization rates was significantly lower in affected cells compared to than in unaffected cells ( $P = 0.046$ , as determined by t-test).

25

#### **Example 7**

##### **Lithium-Induced Changes In Membrane Potential**

The effect of the addition of lithium, a clinically proven mood stabilizer, on changes in membrane potential in K<sup>+</sup>-free buffer and in K<sup>+</sup>-containing buffer was measured. Cells were incubated with or without lithium chloride (LiCl 20 mM) for 2 hours in K<sup>+</sup>-free buffer or in K<sup>+</sup>-containing buffer.

A constant sample volume of each cell line was added to an equal volume of each buffer containing an equal volume of dye. Thus, the K<sup>+</sup>-free buffer and the K<sup>+</sup>-containing buffer contained an equal number of 5 cells from each culture sample and an equal concentration of dye.

The membrane potential of these cells was determined by measuring the fluorescence intensity as described above. The intensity ratio (membrane 10 potential with lithium/membrane potential without lithium) was compared to the intensity ratio in K<sup>+</sup>-containing buffer and/or in K<sup>+</sup>-free buffer. The relative intensity ratio was obtained by dividing the intensity ratio in K<sup>+</sup>-free buffer by the 15 intensity ratio in K<sup>+</sup>-containing buffer. As shown in Figure 11, the relative intensity ratio (ratio in K<sup>+</sup>-free buffer with and without lithium/ratio in K<sup>+</sup>-containing (regular) buffer with and without lithium) of affected cells was significantly higher 20 than that of unaffected cells by t-test ( $P = 0.04$ ).

#### **Example 8**

##### Open Clinical Trials

A pilot clinical trial was conducted using 25 blood samples from 12 clinically affected bipolar patients and 11 unaffected controls. The medical histories of the patients were known to the investigator measuring the membrane potentials; therefore, the trials described in this example were 30 open trials. The patients ranged from 19 years to 77 years in age, and included males and females from different ethnic groups. Similarly, the controls ranged from 25 to 67 including males and females

from different ethnic groups. The patients were all hospitalized following a confirmed manic episode. During the drawing of samples, all patients were on mood stabilizers, either lithium or valproate.

5 Because the volume of blood required to collect enough lymphocytes was beyond that permitted by the current protocol, the membrane potential of whole blood cells was determined. The volume of whole blood required was approximately 1 ml. The blood  
10 samples were kept on ice until they were stored at 4°C. All of the samples were tested within 48 hours.

Whole blood samples drawn from patients and controls were suspended in K<sup>+</sup>-containing buffer without ethacrynat and in K<sup>+</sup>-free buffer with 30μM  
15 ethacrynat or with 100 mM sorbitol. The fluorescent dye DiOC<sub>6</sub>(3) was added to both suspensions and incubated for 30 minutes. The cell suspensions loaded with the dye were centrifuged, drained and resuspended in the respective buffers. The  
20 fluorescence intensity was measured for 10 seconds and the ratio of the intensity in K<sup>+</sup>-free buffer to the intensity in K<sup>+</sup>-containing buffer was calculated.

Tables 5 and 6 below present the statistical analysis of the data generated in the trials.

**TABLE 5**

Clinical Trials (Open Samples)  
 Normality Test: Passed ( $P > 0.050$ )  
 5 Equal Variance Test: Passed ( $P = 0.523$ )

| Group Name | N   | Missing | Mean  | Std Dev | SEM     |
|------------|-----|---------|-------|---------|---------|
| Controls   | 98  | 0       | 0.893 | 0.0485  | 0.00489 |
| Bipolars   | 109 | 0       | 0.780 | 0.0521  | 0.00499 |
| Difference |     |         | 0.114 |         |         |

10  $t = 16.204$  with 205 degrees of freedom. ( $P = <0.001$ )

15 95 percent confidence interval for difference of means: 0.0999  
to 0.128

20 The difference in the mean values of the two groups is greater  
than would be expected by chance; there is a statistically  
significant difference between the input groups ( $P = <0.001$ ).

Power of performed test with alpha = 0.050: 1.000

**TABLE 6**

25 Descriptive Statistics:

| Column   | Size | Missing | Mean  | Std Dev | Std. Error | C.I. of Mean |
|----------|------|---------|-------|---------|------------|--------------|
| Controls | 98   | 0       | 0.893 | 0.0485  | 0.00489    | 0.00971      |
| Bipolars | 109  | 0       | 0.780 | 0.0521  | 0.00499    | 0.00990      |

| Column   | Range | Max   | Min   | Median | 25%   | 75%   |
|----------|-------|-------|-------|--------|-------|-------|
| Controls | 0.256 | 1.053 | 0.797 | 0.893  | 0.860 | 0.925 |
| Bipolars | 0.250 | 0.880 | 0.629 | 0.778  | 0.751 | 0.817 |

| Column   | Skewness | Kurtosis | K-S Dist. | K-S Prob. | Sum    | Sum of Squares |
|----------|----------|----------|-----------|-----------|--------|----------------|
| Controls | 0.287    | 0.141    | 0.0418    | 0.863     | 87.558 | 78.457         |
| Bipolars | -0.425   | 0.0710   | 0.0693    | 0.219     | 84.988 | 66.559         |

Table 5 above indicates that these data passed both the normality test and equal variance test.  
 45 Table 6 above shows that the mean value is very close to the median value for each of these groups, indicating that the data are normally distributed. The t-test shows significant variance ( $P << 0.001$ ) among the two groups in open trials, with post-hoc comparisons showing significant differences between controls and bipolar patients.

Figure 12 graphically depicts the data presented in Tables 5 and 6 above, showing the ratio of fluorescence intensities of the control and bipolar groups in K<sup>+</sup>-free buffer containing 30 μM ethacrynat<sup>e</sup> to the fluorescence intensity in K<sup>+</sup>-containing buffer without ethacrynat<sup>e</sup>. The mean fluorescence intensity ratio of the bipolar samples was significantly lower than that of the control samples by t-test.

Figure 13 shows that with bipolar patients the results with sorbitol were comparable to the results with ethacrynat<sup>e</sup>. This figure shows a comparison of fluorescence intensity ratios of bipolar cells using ethacrynat<sup>e</sup> and sorbitol in K<sup>+</sup>-free buffer.

15

#### **Example 9**

##### Blind Clinical Trials

A clinical trial was conducted using blood samples from the following four groups: schizoprenic, bipolar, and unipolar patients, as well as controls. The medical histories of these patients were unknown to the investigator measuring the membrane potentials; therefore, the trials described in this example were blind trials. The sample size was determined to be 5 with a power of 0.9, an alpha of 0.05, a difference in mean of 0.08, and a standard deviation of 0.03. However, more than 10 samples for each category were tested for a total sample size of 59. If a patient matched a diagnosis, structured clinical interviews according to DSM-IV guidelines were performed.

Whole blood samples drawn from patients and controls were suspended both in K<sup>+</sup>-containing buffer

and in K<sup>+</sup>-free buffer with 30μM ethacrynat or with 100 μM sorbitol. The fluorescent dye DiOC<sub>6</sub>(3) was added to both suspensions and incubated for 30 minutes. The cell suspensions loaded with the dye were centrifuged, drained and resuspended in the respective buffers. The fluorescence intensity was measured for 10 seconds and the ratio of the intensity in K<sup>+</sup>-free buffer to the intensity in K<sup>+</sup>-containing buffer was calculated.

Tables 7 and 8 below present the statistical analysis of the four groups.

**TABLE 7**

One Way Analysis of Variance      SUMMARY OF BLIND TRIALS

Normality Test: Passed      (P > 0.050)  
 Equal Variance Test: Failed      (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

**Kruskal-Wallis One Way Analysis of Variance on Ranks**

| Group      | N   | Missing | Median | 25%   | 75%   |
|------------|-----|---------|--------|-------|-------|
| Controls   | 173 | 0       | 0.850  | 0.829 | 0.869 |
| Schizophr. | 78  | 0       | 0.863  | 0.838 | 0.895 |
| Bipolars   | 178 | 0       | 0.773  | 0.752 | 0.790 |
| Unipolars  | 72  | 0       | 0.901  | 0.863 | 0.934 |

H = 351.025 with 3 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Dunn's Method):

| Comparison              | Diff of Ranks | Q      | P<0.05 |
|-------------------------|---------------|--------|--------|
| Unipolars vs Bipolars   | 308.546       | 15.260 | Yes    |
| Unipolars vs Controls   | 95.297        | 4.694  | Yes    |
| Unipolars vs Schizophr. | 52.263        | 2.209  | No     |
| Schizophr. vs Bipolars  | 256.283       | 13.037 | Yes    |
| Schizophr. vs Controls  | 43.034        | 2.180  | No     |
| Controls vs Bipolars    | 213.249       | 13.797 | Yes    |

**TABLE 8****Descriptive Statistics: SUMMARY OF BLIND TESTS**

| 5  | <b>Column</b> | <b>Size</b>     | <b>Missing</b>  | <b>Mean</b>  | <b>Std Dev</b>   | <b>Std. Error</b> | <b>C.I. of Mean</b>   |
|----|---------------|-----------------|-----------------|--------------|------------------|-------------------|-----------------------|
|    | Controls      | 173             | 0               | 0.850        | 0.0290           | 0.00220           | 0.00435               |
|    | Schizophr.    | 78              | 0               | 0.870        | 0.0428           | 0.00484           | 0.00964               |
|    | Bipolars      | 178             | 0               | 0.768        | 0.0300           | 0.00225           | 0.00444               |
|    | Unipolars     | 72              | 0               | 0.898        | 0.0486           | 0.00573           | 0.0114                |
| 10 | <b>Column</b> | <b>Range</b>    | <b>Max</b>      | <b>Min</b>   | <b>Median</b>    | <b>25%</b>        | <b>75%</b>            |
|    | Controls      | 0.180           | 0.929           | 0.749        | 0.850            | 0.829             | 0.869                 |
|    | Schizophr.    | 0.200           | 0.994           | 0.794        | 0.863            | 0.838             | 0.895                 |
|    | Bipolars      | 0.164           | 0.830           | 0.667        | 0.773            | 0.752             | 0.790                 |
| 15 | Unipolars     | 0.199           | 1.004           | 0.805        | 0.901            | 0.863             | 0.934                 |
|    | <b>Column</b> | <b>Skewness</b> | <b>Kurtosis</b> | <b>Dist.</b> | <b>K-S Prob.</b> | <b>K-S Sum</b>    | <b>Sum of Squares</b> |
| 20 | Controls      | 0.0895          | 0.607           | 0.0400       | 0.661            | 147.073           | 125.176               |
|    | Schizophr.    | 0.682           | 0.132           | 0.0827       | 0.203            | 67.885            | 59.222                |
|    | Bipolars      | -0.601          | 0.187           | 0.0742       | 0.018            | 136.709           | 105.156               |
|    | Unipolars     | 0.0506          | -0.849          | 0.0762       | 0.362            | 64.645            | 58.210                |

Figure 14 graphically depicts the data presented in Tables 7 and 8 above, showing a summary of these blind test results using whole blood samples. The figure shows the ratio of fluorescence intensities of the sample groups in K<sup>+</sup>-free buffer containing 30 μM ethacrynatetraacetate to the fluorescence intensity in K<sup>+</sup>-containing buffer without ethacrynatetraacetate. The coefficient of variation ranged from 3.5 to 4.5 in all these tests. There were no significant differences among the normals and schizophrenics. However, blood samples from bipolar patients were significantly different from the other three groups, as determined by ANOVA ( $P < 0.001$ ). In particular, the fluorescence intensity ratio of the bipolar samples was significantly lower than that of the other three groups. Furthermore, there was a significant difference between normals and unipolar patients, as determined by ANOVA. In particular, the fluorescence intensity ratio of the unipolar samples was significantly higher than that

of the other three groups. These results are therefore useful in diagnosing unipolar (depressive) patients as well.

5

**Example 10**

Diagnosis of an Individual Patient as Bipolar

An individual patient's cell sample was tested as described herein and six to twelve ratios were calculated. These values were treated as a group and  
10 compared with a normal control group (negative control) and a bipolar group (positive control).

Tables 9 and 10 below present the statistics and the procedure employed for "Patient A." At the bottom of Table 9 there is pair wise comparison of  
15 these groups.

**TABLE 9**

One Way Analysis of Variance  
 Normality Test: Passed ( $P > 0.050$ )  
 Equal Variance Test: Passed ( $P = 0.050$ )

| Group     | Name | N   | Missing | Mean  | Std Dev | SEM     |
|-----------|------|-----|---------|-------|---------|---------|
| Controls  |      | 173 | 0       | 0.850 | 0.0290  | 0.00220 |
| Bipolars  |      | 178 | 0       | 0.768 | 0.0300  | 0.00225 |
| Patient A |      | 12  | 0       | 0.766 | 0.0125  | 0.00359 |

| Source of Variation | DF  | SS    | MS       | F       | P      |
|---------------------|-----|-------|----------|---------|--------|
| Between Groups      | 2   | 0.613 | 0.306    | 360.229 | <0.001 |
| Residual            | 360 | 0.306 | 0.000850 |         |        |
| Total               | 362 | 0.919 |          |         |        |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ( $P = <0.001$ ).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):  
 Overall significance level = 0.05

Comparisons for factor:

| Comparison            | Diff of Means | t      | Unadjusted P | Critical Level | Significant? |
|-----------------------|---------------|--------|--------------|----------------|--------------|
| Controls vs Bipolars  | 0.0821        | 26.372 | 0.000        | 0.017          | Yes          |
| Controls vs Patient A | 0.0844        | 9.699  | 0.000        | 0.025          | Yes          |
| Bipolars vs Patient A | 0.00233       | 0.268  | 0.789        | 0.050          | No           |

**TABLE 10**

Descriptive Statistics:

| Column    | Size | Missing | Mean  | Std Dev | Std. Error | C.I. of Mean |
|-----------|------|---------|-------|---------|------------|--------------|
| Controls  | 173  | 0       | 0.850 | 0.0290  | 0.00220    | 0.00435      |
| Bipolars  | 178  | 0       | 0.768 | 0.0300  | 0.00225    | 0.00444      |
| Patient A | 12   | 0       | 0.766 | 0.0125  | 0.00359    | 0.00791      |

| Column    | Range  | Max   | Min   | Median | 25%   | 75%   |
|-----------|--------|-------|-------|--------|-------|-------|
| Controls  | 0.180  | 0.929 | 0.749 | 0.850  | 0.829 | 0.869 |
| Bipolars  | 0.164  | 0.830 | 0.667 | 0.773  | 0.752 | 0.790 |
| Patient A | 0.0394 | 0.787 | 0.748 | 0.763  | 0.755 | 0.777 |

| Column    | Skewness | Kurtosis | K-S Dist. | K-S Prob. | Sum     | Sum of Squares |
|-----------|----------|----------|-----------|-----------|---------|----------------|
| Controls  | 0.0895   | 0.607    | 0.0400    | 0.661     | 147.073 | 125.176        |
| Bipolars  | -0.601   | 0.187    | 0.0742    | 0.018     | 136.709 | 105.156        |
| Patient A | 0.347    | -1.092   | 0.138     | 0.665     | 9.188   | 7.037          |

As shown in Tables 9 and 10 above, there was a significant difference between controls and the patient. However, there was no significant difference between the patient and the bipolar group. From this, Patient A was diagnosed as bipolar.

Figure 15 graphically depicts an example of the use of ANOVA in the diagnosis of Patient A as bipolar. The ratio of fluorescence intensities and therefore membrane potentials in K<sup>+</sup>-free buffer containing 30 μM ethacrynatate was compared to the fluorescence intensity in K<sup>+</sup>-containing buffer without ethacrynatate. The fluorescence intensity ratio of the patient's cell sample was significantly lower than that of the controls, but was not significantly different from that of the bipolars.

#### **Example 11**

##### Diagnosis of an Individual Patient as Unipolar

An individual patient's cell sample is tested as described herein and six to twelve ratios are calculated. These values are treated as a group and compared with a normal control group (negative control) and a unipolar group (positive control), in a manner similar to that described above in Example 10 for diagnosing an individual patient as bipolar.

The ratio of fluorescence intensities and therefore membrane potentials in K<sup>+</sup>-free buffer containing 30 μM ethacrynatate are compared to the fluorescence intensity in K<sup>+</sup>-containing buffer without ethacrynatate.

A patient is diagnosed as unipolar when there is a significant difference between controls and the

patient (i.e., the fluorescence intensity ratio of the patient's cell sample is significantly higher than that of the controls) and/or there is no significant difference between the unipolar group 5 and the patient. ANOVA is used in the diagnosis of a patient as unipolar.

**Example 12**

Specificity and Sensitivity of the Diagnostic Whole

10                   Blood Tests

Specificity is the ability to identify those who do not have the disease (Dawson et al, "Basic and Clinical Biostatistics", Third Edition, Lange Medical Books/McGraw-Hill, New York (2002)). As 15 shown below in Table 11, the specificity in identifying controls was 100% in the blind trials described above in Example 9. Out of a control population (those with no known mental illness) of 18, all were diagnosed as negative for a bipolar disorder. The specificity for the overall non- 20 bipolar (including schizophrenics and unipolars) population of 39 was 85% (33 out of 39) in the above-described blind trials. Among the schizophrenic patients, the test diagnosed 8 out of 25 10 correctly, indicating a specificity of 80% in the above-described blind trials. The specificity for the unipolar patients was 64% (seven out of eleven) in the above-described blind trials.

Sensitivity is defined as a test's ability to 30 detect a disease among patients who actually have the disease (Dawson et al, *supra*). In the above-described blind trials, among the population of 20 bipolar patients (which were determined according to

examination using the DSM IV), 14 were true positives. Hence the sensitivity of the test was 70%. However, doubts about the accuracy of DSM IV-based diagnoses of a bipolar disorder make the estimate of sensitivity unclear.

Consideration was given to whether patients on lithium and patients with diabetes during the blood draw could generate false diagnoses. Of the true positives, one patient was on lithium and 2 were diabetic. Of the false negatives, 2 patients were on lithium and 4 were diabetic. Lithium and diabetes did not have an effect on false positives and true negatives. Thus, lithium and diabetes did not appear to play a role on these tests.

15

**TABLE 11****Summary Of Blind Tests For Bipolar Disorder**

|                       |    |
|-----------------------|----|
| Total samples tested: | 59 |
| Total positives:      | 20 |
| 20 Total negatives:   | 39 |

**Diagnostic Blind Test Results**

|                         |      |
|-------------------------|------|
| 25 Total negatives      | 39   |
| True negatives          | 33   |
| Specificity             | 85%  |
| 30 Total controls       | 18   |
| True controls           | 18   |
| Specificity             | 100% |
| 35 Total schizophrenics | 10   |
| True schizophrenics     | 8    |
| Specificity             | 80%  |
| 40 Total unipolars      | 11   |
| True unipolars          | 7    |
| Specificity             | 64%  |
| Total bipolars          | 20   |
| True positives          | 14   |
| Sensitivity             | 70%  |

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof. All such obvious and foreseeable changes and modifications are intended to be encompassed by the following claims.